

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

*In re* Application of:

Donald W. KUFE and Surender  
KHARBANDA

Serial No.: 10/598,295

Filed: April 5, 2007

For: MUC1 ANTAGONIST ENHANCEMENT  
OF DEATH RECEPTOR LIGAND-  
INDUCED APOPTOSIS

Group Art Unit: 1635  
Examiner: McGarry, Sean  
Atty. Dkt. No.: GENU:006US  
Confirmation No.: 2459

CERTIFICATE OF ELECTRONIC TRANSMISSION 37 C.F.R. § 1.8	
I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:	
September 23, 2010	_____ Date Steven Highlander

DECLARATION OF DONALD W. KUFE UNDER 37 C.F.R. §1.132

Commissioner for Patents  
P. O. Box 1450  
Alexandria, VA 22313-1450

I, Donald W. Kufe, do declare that:

1. I am a United States citizen residing at 179 Grove St., Wellesley, MA, 02482, and the first named inventor of the above-referenced U.S. patent application.
2. I have reviewed the Office Action dated March 25, 2010, that is related to the above-referenced application and understand that the examiner has rejected the pending claims as being obvious in view of papers by Dobie and Tuschl. I understand that the Examiner's position is largely based on an assertion that the end results of antisense technology and RNA interference technology are the same, and that therefore a person of

ordinary skill in the field of the invention would be motivated to use RNA interference to interfere with MUC-1 expression, as taught by Tuschl, based on a Dobie that teaches antisense technology against the same target. I disagree with the Examiner's position.

3. As set forth in Bumcrot *et al.* (*Nature Chemical Biology* 2(12):711-719, 2006), “[i]n RNAi, the target mRNA is enzymatically cleaved, leading to decreased abundance of the corresponding protein.” *Id.* at page 711. Further, the interference RNA is a “double-stranded RNA.” *Id.* at page 711, and FIG. 1. In RNA interference, long double-stranded RNA (dsRNA) is cleaved into small interfering RNA (siRNA). *Id.* at page 712, and FIG. 1. This mechanism is distinct from gene inhibition using antisense RNA, where the antisense RNA is a single-stranded RNA molecule. Thus, “interference RNA” is distinct from “antisense RNA.”
4. Miyagishi *et al.* (*Antisense and Nucleic Acid Drug Development* 13:1-7, 2003) compared the effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. The targets were six sites in the firefly gene for luciferase. Results showed that there were significant differences in the suppressive effects at each of the target sites. See page 5, left column, and FIG. 2A and FIG. 3. As can be seen from FIG. 2A and FIG. 3, the correlation coefficient between the results for antisense ODN's and siRNA was low (0.42).
5. Therefore, a person of ordinary skill in the field, who would have been familiar with Miyagishi *et al.*, would have understood that the effects of antisense technology are not necessarily the same as the with RNA interference and that successful downregulation of a gene with antisense technology does not necessarily predict that the same gene can be downregulated using RNA interference.

6. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

9/14/2010  
Date

Donald W. Kufe  
Donald W. Kufe

# RNAi therapeutics: a potential new class of pharmaceutical drugs

David Bumcrot, Muthiah Manoharan, Victor Koteliansky & Dinah W Y Sah

The rapid identification of highly specific and potent drug candidates continues to be a substantial challenge with traditional pharmaceutical approaches. Moreover, many targets have proven to be intractable to traditional small-molecule and protein approaches. Therapeutics based on RNA interference (RNAi) offer a powerful method for rapidly identifying specific and potent inhibitors of disease targets from all molecular classes. Numerous proof-of-concept studies in animal models of human disease demonstrate the broad potential application of RNAi therapeutics. The major challenge for successful drug development is identifying delivery strategies that can be translated to the clinic. With advances in this area and the commencement of multiple clinical trials with RNAi therapeutic candidates, a transformation in modern medicine may soon be realized.

RNAi is a fundamental cellular mechanism for silencing gene expression that can be harnessed for the development of new drugs<sup>1,2</sup>. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that are difficult to modulate selectively with traditional pharmaceutical approaches involving small molecules or proteins. Consequently, RNAi therapeutics as a drug class have the potential to exert a transformational effect on modern medicine. In RNAi, the target mRNA is enzymatically cleaved, leading to decreased abundance of the corresponding protein, and specificity is a key feature of the mechanism. Synthetic small interfering RNAs (siRNAs) leverage the naturally occurring RNAi process in a manner that is consistent and predictable with regard to extent and duration of action. In addition, viral delivery of short hairpin RNAs (shRNAs) represents an alternative strategy for harnessing RNAi. Both nonviral delivery of siRNAs and viral delivery of shRNAs are being advanced as potential RNAi-based therapeutic approaches.

In this review, we provide an overview of the molecular mechanism of RNAi; the *in silico* design of siRNAs and shRNAs that are specific for a target of interest, in the context of current concepts relating chemical structure to specificity and potency; the use of chemical modifications that confer stability against exo- and endonucleases present in biological fluids and tissues; and strategies for facilitating cellular delivery *in vivo* through conjugation, complexation and lipid-based approaches to facilitate cellular uptake. We summarize the numerous publications to date demonstrating the robust efficacy of RNAi in animal models of human disease upon direct (local) as well as systemic administration. These proof-of-concept studies support RNAi as the basis for a new therapeutic approach that has the potential to change the treatment of human disease. Most importantly, as we will discuss, clinical trials have

recently commenced, with RNAi therapeutic candidates under study for treatment of age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection.

## Molecular mechanism of RNAi

The RNase Dicer initiates RNAi by cleaving double-stranded RNA substrates into small fragments of about 21–25 nucleotides in length (Fig. 1). These siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex (RISC; Fig. 1). Biochemical analysis identified Argonaute 2 (Ago2) as the protein in RISC responsible for mRNA cleavage<sup>3</sup>, and the crystal structure of RNA-bound Ago2 has been reported, revealing key interactions<sup>4</sup>.

Before RISC activation, the sense (nonguide) strand of the siRNA duplex is cleaved by Ago2, in the same manner as it cleaves mRNA substrates<sup>5,6</sup>. Preventing sense-strand cleavage by chemical modification can reduce siRNA potency *in vitro*; however, experimental context is important, as siRNAs with highly stabilized (uncleavable) sense strands can be highly active.

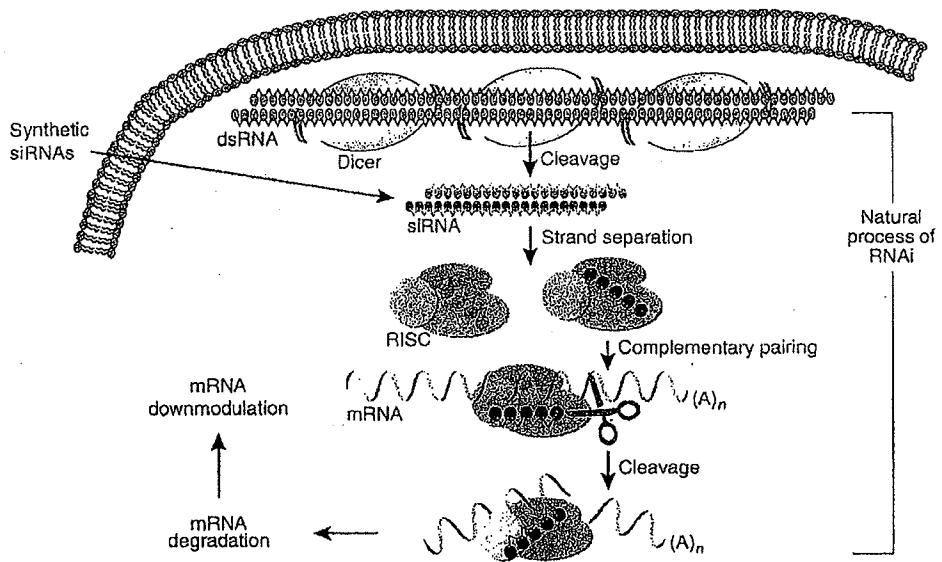
## Role of chemical modifications

Small-molecule pharmaceutical drugs, almost without exception, meet the 'Lipinski Rules', criteria including high lipophilicity and molecular weight of not more than 500. In sharp contrast, siRNAs naturally lack these drug-like properties owing to their large size (two turns of a nucleic acid double helix), nearly 40 anionic charges due to the phosphodiester backbone, and high molecular weight (over 13 kDa). In aqueous solution, with their sugar-phosphate backbone exposed to water, siRNAs are extremely hydrophilic and heavily hydrated. Furthermore, siRNAs are unstable in serum as a result of degradation by serum nucleases, contributing to their short half-lives *in vivo*<sup>7</sup>. Although the molecular weight of siRNAs cannot be reduced, these molecules can be made more 'drug-like' through judicious use of chemical modification to the sugars, backbone or bases of the oligoribonucleotides.

Chemically modified siRNA duplexes have been evaluated in cell-based assays and in animal models. The modifications discussed are

Alnylam Pharmaceuticals, Inc., 300 Third Street, Cambridge, Massachusetts 02142, USA. Correspondence should be addressed to D.W.Y.S. (dsah@alnylam.com).

Published online 15 November; doi:10.1038/nchembio839



**Figure 1** Cellular mechanism of RNA interference. Long double-stranded RNA (dsRNA) is cleaved, by the enzyme Dicer, into small interfering RNA (siRNA). These siRNAs are incorporated into the RNA-induced silencing complex (RISC), where the strands are separated. The RISC containing the guide or antisense strand seeks out and binds to complementary mRNA sequences. These mRNA sequences are then cleaved by Argonaute, the enzyme within the RISC, responsible for mRNA degradation, which leads to mRNA down-modulation. A, adenosine.

shown in Figure 2. Stability against nuclease degradation has been achieved by introducing a phosphorothioate (P=S) backbone linkage at the 3' end for exonuclease resistance and 2' modifications (2'-OMe, 2'-F and related) for endonuclease resistance<sup>8–10</sup>. An siRNA motif, consisting entirely of 2'-O-methyl and 2'-fluoro nucleotides, has enhanced plasma stability and increased *in vitro* potency. At one site, this motif shows >500-fold improvement in potency over the unmodified siRNA<sup>11</sup>. Using phosphatase and tensin homolog (PTEN) as a target, the effect of 2' sugar modifications such as 2'-fluoro-2'-deoxyribose (2'-F), 2'-O-methyl (2'-O-Me) and 2'-O-(2-methoxyethyl) (2'-O-MOE) in the guide and nonguide strands was evaluated in HeLa cells. The activity depends on the position of the modification in the guide-strand sequence. The siRNAs with modified residues at the 5' end of the guide strand seem to be less active than those modified at the 3' end. The 2'-F sugar is generally well tolerated on the guide strand, whereas the 2'-O-MOE modification results in loss of activity regardless of placement position in the construct. The incorporation of 2'-O-Me and 2'-O-MOE in the nonguide strand of siRNA does not have a notable effect on activity<sup>12</sup>. Sugar modifications such as 2'-OMe, 2'-F and locked nucleic acid (LNA, with a methylene bridge connecting 2' and 4' carbons) seem to be able to reduce the immunostimulatory effects of siRNAs (see below).

Duplexes containing the 4'-thioribose modification have a stability 600 times greater than that of natural RNA<sup>13</sup>. Crystal structure studies reveal that 4'-thioriboses adopt conformations very similar to the C3'-endo pucker observed for unmodified sugars in the native duplex<sup>14</sup>. Stretches of 4'-thio-RNA were well tolerated in both the guide and non-guide strands. However, optimization of both the number and the placement of 4'-thioribonucleosides is necessary for maximal potency. These optimized siRNAs are generally equipotent with or superior to native siRNAs and show increased thermal and plasma stability. Furthermore, substantial improvements in siRNA activity and plasma stability have been achieved by judicious combination of 4'-thioribose with 2'-OMe and 2'-O-MOE modifications<sup>15</sup>.

As mentioned, phosphorothioate (P=S) modifications are generally well tolerated on both strands and provide improved nuclease resistance. The 2',5'-phosphodiester linkages seem to be tolerated in the nonguide but not the guide strand of the siRNA<sup>16</sup>. In the borano-phosphate linkage, a nonbridging phosphodiester oxygen is replaced by an isoelectronic borane (BH<sub>3</sub><sup>-</sup>) moiety. Boranophosphate siRNAs have been synthesized by enzymatic routes using T7 RNA polymerase and a boranophosphate ribonucleoside triphosphate in the transcription reaction. Boranophosphate siRNAs are more active than native siRNAs if the center of the guide strand is not modified, and they may be at least ten times more nuclease resistant than unmodified siRNAs<sup>17,18</sup>.

siRNA duplexes containing the 2,4-difluorotolyl ribonucleoside (rF) were synthesized to evaluate the effect of noncanonical nucleoside mimetics on RNA interference. Thermal melting analysis showed that the base pair between rF and adenosine is destabilizing relative to a uridine-adenosine pair, although it is slightly less destabilizing than other mismatches. The crystal structure of a duplex containing rF-adenosine pairs shows local structural variations relative to a canonical RNA helix. As the fluorine atoms cannot act as hydrogen bond acceptors and are more hydrophobic than uridine, a well-ordered water structure is not seen around the rF residues in both grooves. Rapid amplification of 5' complementary DNA ends (5'-RACE) analysis confirms cleavage of target mRNA opposite to the rF placement site<sup>19,20</sup>.

Certain terminal conjugates have been reported to improve or direct cellular uptake. For example, siRNAs conjugated with cholesterol improve *in vitro* and *in vivo* cell permeation in liver cells<sup>6</sup>. As described below, cholesterol and an RNA aptamer conjugation show promise in animal models.

#### Design considerations for potency and specificity

Critical design concerns in the selection of siRNA duplexes for therapeutic use are potency and specificity. There are two major considerations

with regard to siRNA specificity: 'off-targeting' due to silencing of genes sharing partial homology with the siRNA, and 'immune stimulation' due to the engagement of components of the innate immune system by the siRNA duplex. A combination of bioinformatics methods, chemical modification strategies and empirical testing is required to address these issues.

Concomitant with the first description of the structure of active siRNAs, a set of 'rules' was proposed for selecting potent siRNA duplex sequences<sup>21,22</sup>. Several groups have subsequently developed more sophisticated extensions of these largely empirical criteria, leading to the development of algorithms for siRNA design<sup>23,24</sup>. Recent biochemical studies of the molecular mechanism of RNA interference have highlighted some key features of potent siRNA duplexes (Fig. 3). Most notably, it has been found that the efficiency with which the guide strand is incorporated into the RISC complex is perhaps the most important factor determining siRNA potency. Because siRNA duplexes are symmetric, the question arose of how the RISC machinery is able to determine which strand to use for target silencing. Insight into this enigma came from careful analyses of microRNAs (miRNAs), the endogenous counterparts of siRNAs. Examination of the sequences of a large number of vertebrate and invertebrate miRNA precursor sequences showed that the predicted thermodynamic stabilities of the two ends of the duplex are unequal<sup>25,26</sup>. Specifically, calculating the  $\Delta G$  for the several base pairs at each end of the duplex revealed that the 5' end of the mature miRNA pairs less tightly with the carrier strand than does the 3' end. In short, miRNA precursors show thermodynamic asymmetry. It was hypothesized that components of the RISC machinery select the guide strand based on this asymmetry.

Experimental evidence supporting the asymmetry hypothesis has been derived from studies using chemically synthesized siRNAs in transfection experiments. Through an elegant assay in which each strand of the siRNA targets a different reporter gene, Schwarz *et al.* were able to quantify the relative efficiency of RISC incorporation for each of the two strands<sup>25</sup>. They found that the RISC machinery preferentially incorporates the strand whose 5' end binds less tightly with the other strand. In fact, strand selection could be switched by making a single nucleotide substitution at the end of the duplex to alter relative binding of the ends. A similar conclusion was reached by another group based on *in vitro* screening of a large collection of siRNAs with varying potency<sup>26,27</sup>. Thus, designing siRNAs with relatively weaker base pairing at the 5' end of the desired guide strand may increase the likelihood of obtaining a potent duplex.

The issue of off-target silencing has been the subject of intensive study in a number of different laboratories over the past several years. Transcriptional profiling studies have confirmed that siRNA duplexes can potentially silence multiple genes in addition to the intended target. As expected, genes in these so called off-target 'signatures' contain regions that are complementary to one of the two strands in the siRNA duplex<sup>28–30</sup>. More detailed bioinformatic analyses have revealed that the regions of complementarity are most often found in the 3' UTRs of the off-target genes<sup>31</sup>. This immediately suggested a microRNA-like mechanism, because miRNAs generally interact with the 3' UTR region of their targets. Evidence in support of this concept came from a closer look at the determinants of siRNA off-targeting. It was discovered that sequence complementarity between the 5' end of the guide strand and the mRNA is the key to off-target silencing<sup>31,32</sup>. The critical nucleo-

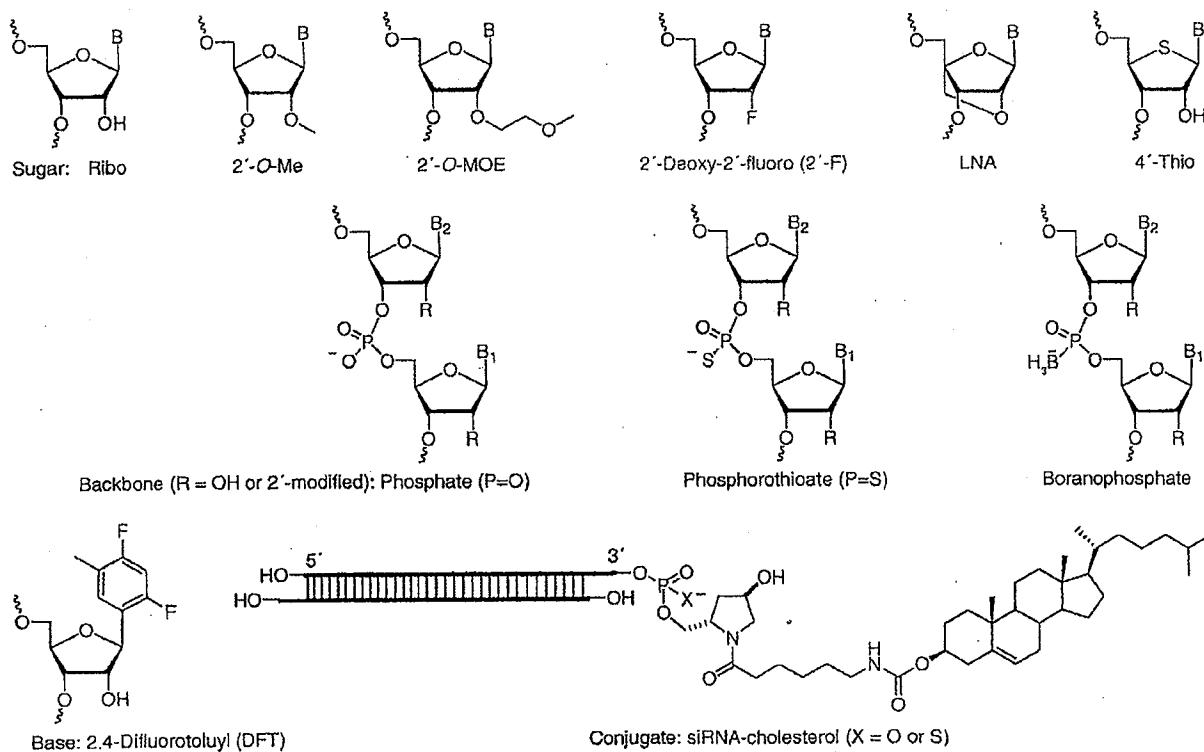


Figure 2 Chemical modifications of siRNAs. Shown are structures of sugar, backbone and base modifications and of the cholesterol conjugate.

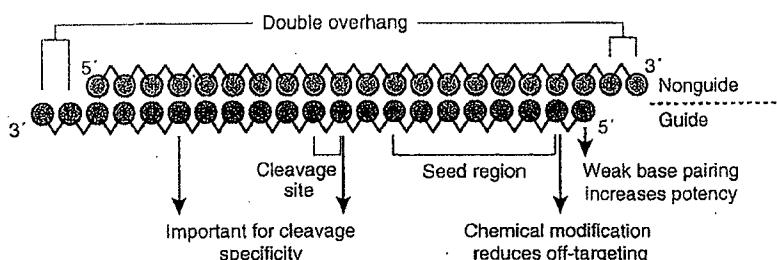


Figure 3 Critical nucleotide positions in siRNAs. Nucleotides that are important for potency, mRNA recognition, mRNA cleavage and cleavage specificity, including minimization of off-targeting, are shown.

tides were found to be positions 2–8, counting from the 5' end of the guide strand (Fig. 3). This corresponds to the so-called 'seed region' of miRNAs, which has been shown to determine miRNA specificity<sup>33</sup>.

Two strategies for avoiding seed region-mediated off-targeting can be envisioned. The first is simply to ensure that nucleotides complementary to positions 2–8 of the guide strand are unique to the intended target. Though theoretically possible, this approach may prove impractical, as the universe of possible seed-region heptamers is only 16,384 distinct sequences. Even if the homology is restricted to the 3' UTR, it may prove difficult to identify siRNA duplexes satisfying the criteria of potency and specificity. As one alternative, recent published work has reported that off-targeting can be substantially reduced by chemical modification of nucleotides within the seed region<sup>34</sup>. Specifically, the introduction of a 2'-O-Me modification into nucleotides within the seed region was shown to inactivate the off-target activity of the siRNA without compromising silencing of the intended mRNA. In fact, introduction of the modification at a single nucleotide position (position 2, Fig. 3) is sufficient to suppress the majority of off-targeting. The mechanism, anticipated by recently published crystal structure data, appears to involve perturbation of RISC interaction with the modified nucleotide.

Interactions outside of the seed region can also substantially affect siRNA specificity. Although the seed region seems to be critical for mRNA recognition, notable mRNA cleavage requires more extensive base pairing between the siRNA and the target<sup>32</sup>. In a recent study, Schwarz *et al.* designed siRNAs capable of distinguishing between mRNA targets that differ by only one nucleotide<sup>35</sup>. They showed that target selectivity depends on the location of the mismatch between the siRNA and the mRNA. Whereas positioning the mismatch within the seed region imparts a certain degree of selectivity, positioning the mismatch further 3' in the guide strand (especially at positions 10 and 16, Fig. 3) produces highly discriminatory siRNAs. The authors hypothesized that mismatches at these positions are particularly disruptive to the helical structure of the siRNA–mRNA complex required for target cleavage.

A second mechanism whereby siRNA duplexes can induce unintended effects is through stimulation of the innate immune system in certain specialized immune cell types. It has been demonstrated that siRNA duplexes harboring distinct sequence motifs can engage Toll-like receptors (TLRs) in plasmacytoid dendritic cells, resulting in increased production of interferon<sup>36</sup>. Such immune stimulation could pose a significant problem in a therapeutic setting. This phenomenon is reminiscent of the results of earlier studies with DNA antisense oligonucleotides in which distinct sequences (so-called CpG motifs) were shown to be immunostimulatory<sup>37</sup>. Subsequent studies established that TLR-9, the receptor for unmethylated CpG-containing pathogen DNA, is the innate

immune regulator engaged by antisense oligonucleotides<sup>38</sup>. In the case of siRNAs, it seems to be TLR-7 that is the mediator of immune stimulation<sup>36</sup>.

Several possible strategies exist for avoiding immune stimulation by siRNA duplexes, including avoidance of the offending sequences during siRNA design and chemical modification to inactivate the motifs. The former approach is not feasible at present because the full spectrum of stimulatory motifs has not been identified. Evidence supporting the latter approach comes from studies in which chemical modifications at the 2' position of nucleotides within putative TLR-7-interacting sequences eliminate immune stimulation

without compromising silencing activity<sup>36,39</sup>. Another possibility would be to use siRNA delivery strategies that avoid the cell types responsible for immune stimulation.

Prediction of the nucleotide sequence and chemical modifications required to yield an ideal siRNA duplex remains a work in progress. Still, the recent advances described above have allowed the development of design algorithms that greatly increase the likelihood of success. It is nonetheless important to note that the relevance of *in vitro* measurements of potency and specificity to *in vivo* activity in a therapeutic setting has yet to be established. For example, the spectrum of off-target genes identified in tissue culture studies can differ depending on the method by which siRNAs are introduced into cells<sup>40</sup>. Also, the induction of an innate immune response by certain siRNA sequences is cell type dependent<sup>41</sup>. At present, the most prudent and robust strategy is to synthesize and screen a substantial library of siRNA duplexes for each target of interest (perhaps even 'tiling' the entire messenger RNA) to identify the most promising candidates.

#### Proof of concept for local RNAi in animal models

During the past several years, numerous studies have been published demonstrating efficacious silencing of disease genes by local administration of siRNAs or shRNAs in animal models of human disease. Both exogenous and endogenous genes have been silenced, and promising *in vivo* results have been obtained across multiple organs and tissues. Efficacy has been demonstrated for viral infection (respiratory and vaginal), ocular disease, disorders of the nervous system, cancer and inflammatory bowel disease (Fig. 4). An important aspect of these proof-of-concept studies is that they have supported the expected high specificity of RNAi.

Local RNAi can protect against both respiratory<sup>42,9</sup> and vaginal<sup>43</sup> viral infections. Two reports illustrate efficacious direct delivery of siRNA to the lung in rodent and monkey models of RSV, influenza and severe acute respiratory syndrome (SARS) infection with and without lipid formulation. In mouse models of infection, pulmonary viral titers of RSV and parainfluenza were reduced by more than 99% with intranasal delivery of siRNAs formulated with TransIT-TKO, a cationic polymer-based transfection reagent, targeting RSV and parainfluenza virus, respectively<sup>42</sup>. In addition, siRNA targeting RSV reduced pulmonary pathology, as assessed by respiratory rate, leukotriene induction and inflammation. These positive proof-of-concept studies in mice have led to clinical trials of RNAi therapeutics targeting RSV.

Another system for which there have been multiple examples of efficacious local delivery of siRNA is the eye, where proof of concept has been successfully achieved in animal models of ocular neovascularization and scarring using saline and lipid formulations<sup>44–46</sup>. Intravitreal injection

of siRNA targeting vascular endothelial growth factor (VEGF) receptor-1, formulated in phosphate-buffered saline, was effective in reducing the area of ocular neovascularization by one-third to two-thirds in two mouse models<sup>44</sup>. In addition, siRNAs targeting VEGF and the transforming growth factor- $\beta$  receptor type II, formulated with TransIT-TKO, were injected directly into the mouse eye, resulting in inhibition of laser photocoagulation-induced choroidal neovascularization<sup>45</sup> and latex bead-induced collagen deposition and inflammatory cell infiltration<sup>46</sup>, respectively. As with the lung, multiple siRNA formulations were effective in the eye. These encouraging proof-of-concept studies in animal models have led to clinical trials of siRNAs targeting the VEGF pathway in AMD.

In the nervous system, RNAi has been particularly useful for validating disease targets *in vivo*. Again, several formulations, including saline, polymer complexation and lipid or liposomal formulations, have been efficacious for delivering siRNAs locally to the nervous system in numerous disease models. The simplest mode of delivery is intracerebroventricular, intrathecal or intraparenchymal infusion of naked siRNA formulated in buffered isotonic saline, which results in silencing of specific neuronal molecular mRNA targets in multiple regions of the central and peripheral nervous systems<sup>47–50</sup>. With naked siRNA formulated in buffered isotonic saline, doses of 0.4 mg per day are typically required for effective target gene silencing. Polymer complexation and lipid or liposomal formulations such as polyethylene imine (PEI), iFECT, DOTAP and JetSI/DOPE facilitate cellular uptake and reduce the doses of siRNA required for effective neuronal target silencing *in vivo* to approximately 5–40  $\mu$ g<sup>51–54</sup>.

Local viral delivery of shRNA to the nervous system has been reported *in vivo* with adenoviral, adeno-associated viral (AAV) and lentiviral delivery in normal mice<sup>55</sup> as well as in animal models of spinocerebellar ataxia<sup>56</sup>, Huntington disease<sup>57,58</sup>, amyotrophic lateral sclerosis (ALS)<sup>59,60</sup> and Alzheimer disease<sup>61</sup>, where abnormal, disease phenotypes including behavior and neuropathology were normalized. Notably, all of the *in vivo* studies to date have targeted genes expressed in neurons; it remains to be seen whether silencing *in vivo* can be achieved in other nervous-system cell types such as oligodendrocytes and astrocytes. Moreover, for endogenous neuronal targets, expression of the target gene is typically reduced only partially, and in some cases by as little as 10–20%, yet this modest reduction in mRNA results in a marked effect on the specific behavior appropriate to the targeted gene.

For application to oncology, direct delivery of siRNAs and viral delivery of shRNAs to tumors have been successful in inhibiting xenograft growth in several mouse models. A number of approaches—including lipid-based formulation (TransMessenger<sup>62</sup>) and complexation with PEI<sup>63</sup>, cholesterol-oligoarginine<sup>64</sup>, a protamine-Fab fusion protein<sup>65</sup> and atelocollagen<sup>66,67</sup>—have been shown to facilitate delivery into tumor cells. Notably, these siRNA delivery approaches are effective with several or even a single intratumor injection of siRNA, at microgram doses. Very recently, aptamer-siRNA chimeric RNAs have also been used successfully to facilitate siRNA delivery *in vivo*, resulting in

tumor regression in a xenograft model of prostate cancer<sup>68</sup>. Viral and vector-based delivery of shRNAs directly to the tumor site<sup>69,70</sup> has also been used effectively in mouse models of adenocarcinoma, Ewing sarcoma and prostate cancer. Of the multiple delivery strategies that have been effective in mouse tumor models, the aptamer approach has the potential of substantially simplifying delivery, if an aptamer is available for a tumor-specific receptor such as prostate-specific membrane antigen (PMSA) and the large-scale synthesis of such a construct is feasible.

For inflammatory bowel disease, direct delivery of siRNA targeting tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) with a Lipofectamine formulation has recently been shown to reduce not only TNF- $\alpha$  abundance but also colonic inflammation after administration by enema<sup>71</sup>. This report, together with a study of siRNA targeting herpes simplex virus-2 (ref. 43), suggests that mucosal surfaces are accessible with liposomal siRNA formulations.

#### Proof of concept for systemic RNAi in animal models

Over the past several years, a number of studies have been published demonstrating the silencing of disease genes by systemic administration of siRNAs (Fig. 4; reviewed in refs. 1,72). In some of these studies, silencing of endogenously expressed genes has shown promising *in vivo* results in different disease contexts. For example, efficacy has been demonstrated in mouse models of hypercholesterolemia and rheumatoid arthritis. In other work, systemic RNAi targeting exogenous genes has shown promise in models of viral infection (hepatitis B virus (HBV), influenza virus, Ebola virus) and in tumor xenografts. Critical to the success of most of these studies has been the use of chemical modifications or delivery formulations that impart desirable pharmacokinetic properties to the siRNA duplex and that also promote cellular uptake in tissues.

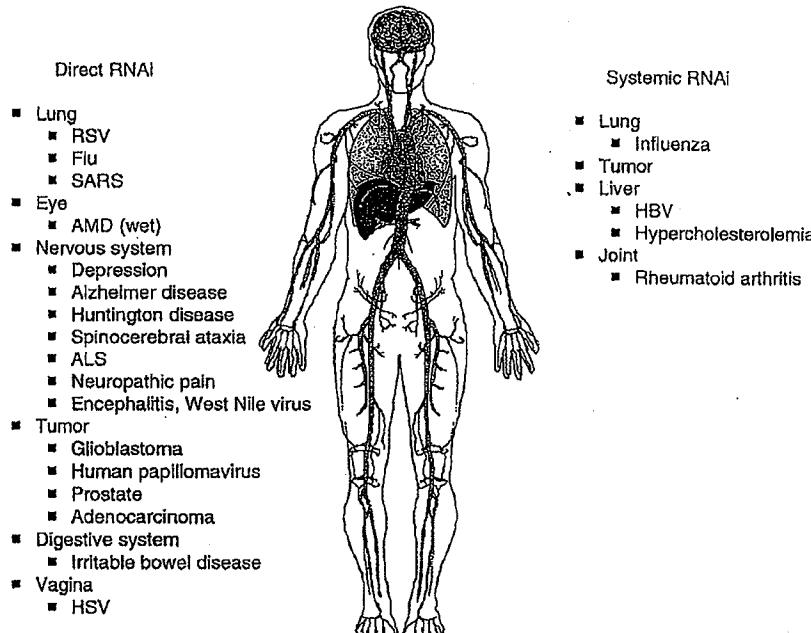


Figure 4 Organs for which RNAi proof of concept has been demonstrated. Direct RNAi represents local delivery of RNAi, and has been carried out successfully to specific tissues or organs, including lung, eye, the nervous system, tumors, the digestive system and vagina. Systemic RNAi represents intravenous delivery of RNAi and has been carried out successfully to lung, tumors, liver and joint. Specific disease models are indicated where efficacy was achieved.

In 2004, Soutschek *et al.* demonstrated effective silencing of the apolipoprotein apoB in mice by intravenous administration of cholesterol-conjugated siRNA duplexes<sup>6</sup>. Three daily injections of cholesterol-conjugated siRNA at a dose of 50 mg kg<sup>-1</sup> resulted in silencing of the apoB mRNA by 57% and 73%, respectively, in the liver and jejunum, the two principal sites for apoB expression. The mechanism of action was proven, by 5'-RACE, to be RNAi mediated. This mRNA silencing produced a 68% reduction in apoB protein abundance in plasma and a 37% reduction in total cholesterol. These therapeutically relevant findings were completely consistent with the known function of apoB in lipid metabolism. Cholesterol conjugation imparted critical pharmacokinetic and cellular uptake properties to the siRNA duplex.

Further advances in systemic RNAi with optimized delivery have recently been reported. Recently, Zimmermann *et al.* made use of siRNA duplexes formulated in stable nucleic acid lipid particles (SNALPs<sup>39</sup>) to recapitulate the silencing of apoB in mice<sup>73</sup>. In rodent studies, the silencing produced by a single dose of SNALP-formulated siRNA at 2.5 mg kg<sup>-1</sup> was greater than that reported in the earlier study using cholesterol-conjugated siRNAs. More importantly, therapeutic silencing of apoB was also demonstrated in nonhuman primates. A single dose of 2.5 mg kg<sup>-1</sup> siRNA encapsulated in the SNALP formulation reduced apoB mRNA in the livers of cynomolgus monkeys by more than 90%. As in the mouse experiments, apoB silencing was accompanied by substantial reductions in serum cholesterol (>65%) and low-density lipoproteins (>85%). Furthermore, silencing was shown to last for at least 11 d after a single dose. In addition, the treatment seemed to be well tolerated, with transient increases in liver enzymes as the only reported evidence of toxicity. This primate study represented an important step forward in the development of systemic RNAi for therapeutic applications. Moreover, the general applicability of SNALP formulations for hepatic delivery of siRNA has been demonstrated in animal models of HBV and Ebola virus infection<sup>74,75</sup>.

In mouse tumor xenograft models, the efficacy of systemic RNAi has been demonstrated using a variety of delivery strategies (reviewed in refs. 1,2,76). Systemically delivered cationic cardiolipin liposomes containing siRNA specific for Raf-1 inhibit tumor growth in a xenograft model of human prostate cancer<sup>77</sup>. Vascular endothelial growth factor receptor-2 (VEGF-R2)-targeting siRNAs complexed with self-assembling nanoparticles consisting of polyethylene glycol-conjugated (PEGylated) PEI with an Arg-Gly-Asp peptide attached at the distal end of the PEG accumulate in tumors and cause inhibition of VEGF-R2 expression. Intravenous administration of these complexes into tumor-bearing mice inhibits both tumor angiogenesis and growth rate<sup>78</sup>. Simpler PEI formulations have also shown efficacy in xenograft tumor models<sup>79</sup>, as have complexes of siRNA duplexes with atelocollagen. Systemic administration of atelocollagen–siRNA complexes has marked effects on subcutaneous tumor xenografts<sup>66</sup> as well as bone metastases<sup>76</sup>. Another recently described delivery strategy made use of a recombinant antibody fusion protein to achieve cell type-specific delivery. As described above, Song *et al.* fused the nucleic acid binding protein protamine to the C terminus of a fragment antibody (Fab) targeting the HIV-1 envelope protein gp160. After systemic administration, the Fab-protamine fusion was able to deliver an siRNA mixture to mouse melanoma cells engineered to express the envelope protein, leading to substantial inhibition of tumor growth in mice. Tumors derived from cells not expressing the envelope protein were unaffected. In another example of ligand-directed delivery, Hu-Lieskovsky *et al.* made use of transferrin-conjugated nanoparticles to deliver an siRNA targeting the oncogenic EWS-FLI translocation-derived mRNA in a mouse model of metastatic Ewing sarcoma<sup>80</sup>. Removal of the targeting ligand or the use of a control siRNA sequence eliminated the antitumor effects.

### Comparison of different delivery strategies for RNAi

Effective delivery is perhaps the most challenging remaining consideration for successful translation of RNAi to the clinic and to broad use in patients. In the animal studies reviewed above, nonviral and viral approaches, local and systemic administration, and multiple formulations (saline, lipids, and complexes or conjugates with small molecules, polymers, proteins and antibodies) have all been used to achieve efficacy. However, each of these approaches has distinct advantages and disadvantages for clinical translation, which require careful consideration.

Although viral delivery provides the potential advantage that a single administration could lead to durable down-modulation of the targeted pathological protein, a major risk was highlighted recently<sup>81</sup>. With AAV delivery of shRNAs, excessive diversion of the endogenous RNAi mechanism occurred that resulted in pronounced toxicity in mice. Clearly, for all drugs, it is critical to be able to control the level of drug and the duration of drug action, such that the exposure is safe while still being efficacious. In distinct contrast to nonviral delivery of siRNAs, a substantial liability of viral delivery is that it is impossible to fully predict drug exposure, with regard to both amount and timing. In addition, as shRNAs enter the RNAi pathway upstream of therapeutic applications for siRNAs, viral vectors expressing high levels of shRNAs may interfere with endogenous miRNA biogenesis.

The principal considerations for selecting local versus systemic siRNA administration are the doses needed to achieve sufficient drug concentration in the target tissue and the possible effects of the exposure of nontargeted tissues to drug. At one extreme, with certain tissues, efficacy has so far been demonstrated only with local delivery; current formulations may not provide sufficient drug concentration in the target tissue after systemic delivery. However, with other tissues (for example, liver), intravenous doses in the low mg kg<sup>-1</sup> range with liposomal formulation provide robust therapeutic gene silencing. In general, and as with any pharmacologic approach, the doses of siRNA required for efficacy are substantially lower when siRNAs are injected into or near the target tissue than when they are administered systemically. Given the high specificity of siRNAs for their intended molecular target, exposure of nontargeted tissues to drug is an issue only if the molecular target is expressed in nontargeted tissue and has an important role in normal cellular function within that tissue. In these cases, local delivery with more focused exposure might circumvent undesired side effects resulting from systemic delivery.

Liposomes, and lipid complexes or conjugates with small molecules, polymers, proteins and antibodies, have all been used to facilitate delivery of siRNAs to target cells. With these delivery partners, more robust efficacy can be achieved with doses of siRNA that are substantially lower, less frequent or both. For the additional (non-siRNA) components, however, there are associated biological and large-scale manufacturing considerations. Lipids and polymers can have cytotoxic effects that might limit their use in siRNA delivery for particular disease indications and dosing paradigms. However, it seems to be possible to identify lipid-based formulations and dosing regimens for which cytotoxicity is minimal and the risk of histopathology is reduced<sup>51–53</sup>. Small molecules, proteins and antibodies used as conjugates also need to be considered from the standpoint of biological activity. If the endogenous molecule (for example, receptor) with which they interact has an important role in normal physiology, then using this endogenous molecule to potentiate delivery may alter its normal function and produce undesired side effects. In all of these cases, the additional non-siRNA molecule or molecules increase the complexity of manufacturing, particularly at large scale. With scientific and technical advances, these approaches may provide marked enhancements to siRNA delivery with acceptable biological and manufacturing considerations.

Table 1. Therapeutic modalities: pros and cons

Small molecules	Proteins and Antibodies	RNAi
<ul style="list-style-type: none"> <li>■ Antagonism or agonism of target</li> <li>■ Extracellular and intracellular targets</li> <li>■ Not all target classes can be modulated selectively and potently</li> <li>■ Lead ID and optimization slow</li> <li>■ Easy to synthesize</li> </ul>	<ul style="list-style-type: none"> <li>■ Antagonism or agonism of target</li> <li>■ Extracellular targets</li> <li>■ Highly selective and potent</li> <li>■ Lead ID and optimization slow</li> <li>■ Difficult to produce</li> </ul>	<ul style="list-style-type: none"> <li>■ Antagonism only</li> <li>■ All targets, including 'nondruggable targets'</li> <li>■ Highly selective and potent</li> <li>■ Rapid lead ID and optimization</li> <li>■ Easy to synthesize</li> </ul>

Key features of the two major classes of traditional pharmaceutical drugs—small molecules, and proteins and antibodies—are shown for comparison with RNAi as a therapeutic approach.

### Clinical trials with RNAi therapeutics

The first clinical trials with RNAi therapeutics target the VEGF pathway for the wet form of AMD and the RSV genome for treatment of RSV infection; in both cases the initial approach is direct administration of the RNAi therapeutic in a saline formulation. In both these cases, highly validated disease targets are being inhibited with siRNAs. Furthermore, direct administration of siRNAs to the eye and lung for AMD and RSV infection, respectively, maximizes the chances of delivering sufficient and therapeutically relevant concentrations of drug to the tissue of interest. In a phase 2 trial in patients with serious progressive AMD, the siRNA Cands5, targeting VEGF, has been reported to provide dose-related benefits with respect to several endpoints, including near vision and lesion size (<http://www.acuitypharma.com/press/release13.pdf>). Cands5 is also being tested for efficacy against diabetic macular edema in a phase 2 trial that began in early 2006 (<http://www.acuitypharma.com/press/release10.pdf>). The siRNA Sirna-027, targeting VEGF receptor-1, has recently completed phase 1 trials in patients with the wet form of AMD, in whom it was reported to be well tolerated. In addition, it was reported to stabilize or improve visual acuity in a subset of patients (<http://www.sirna.com/wt/page/ocular>). For RSV infection, two phase 1 trials with the siRNA ALN-RSV01 have been completed in over 100 healthy adult volunteers, in one of the largest human studies with an RNAi therapeutic, and ALN-RSV01 was found to be safe and well tolerated (<http://phx.corporate-ir.net/phoenix.zhtml?c=148005&p=irol-newsArticle2&ID=849576&highlight=1>). Additional RNAi therapeutic candidates that are expected to advance into the clinic within the coming year include siRNAs targeting pandemic influenza (<http://www.alnylam.com/therapeutic-programs/programs.asp>) and hepatitis C (Sirna-034; [http://www.sirna.com/wt/page/anti\\_viral](http://www.sirna.com/wt/page/anti_viral)). As these and other trials advance through the clinic in the near future, the exciting potential of siRNAs may be demonstrated.

### Comparison of RNAi with traditional pharmaceutical drugs

As a therapeutic approach, RNAi provides solutions to the major drawbacks of traditional pharmaceutical drugs (Table 1). The principal advantages of RNAi over small-molecule and protein therapeutics are that all targets, including 'non-druggable' targets, can be inhibited with RNAi and that lead compounds can be rapidly identified and optimized. The primary challenge associated with small-molecule drugs is the identification of highly selective and potent compounds—a difficult and time-consuming process that, for some targets, can be unsuccessful. With RNAi, the identification of highly selective and potent sequences is rapid and has been demonstrated with numerous molecular targets

across all molecular classes. With protein and antibody drugs, the main technical challenge is production. For proteins, acceptable cellular production levels are often difficult to achieve. For biologics as a therapeutic class, aggregation continues to be a major issue. In contrast, siRNAs are synthetic and easy to produce from a chemistry standpoint. Of course, with RNAi, by definition, only antagonism of the specific molecular target is possible, whereas small molecules, proteins and antibodies provide an opportunity for agonism of a molecular target. Overall, however, RNAi holds great promise as a therapeutic approach providing a major new class of drugs that will fill a significant gap in modern medicine.

### Conclusions

Significant progress has been made in advancing RNAi therapeutics in a remarkably short period of time. Starting from the discovery that RNAi is mediated by long double-stranded RNA in *Caenorhabditis elegans* by Fire and Mello in 1998<sup>82</sup> and the publication in 2001 by Tuschi and his Max Planck Institute colleagues that synthetic siRNAs can silence target genes in mammalian systems<sup>20</sup>, the relatively short years since have seen an explosion in reports on therapeutic applications that harness RNAi. Clearly, the principal challenge that remains in achieving the broadest application of RNAi therapeutics is the hurdle of delivery. That said, tremendous progress has been made with new conjugation, complexation and lipid-based approaches, although the challenge of siRNA delivery has yet to be solved for all cell types in all organs. Once that challenge is met, the development of RNAi therapeutics will be limited primarily by target validation. It will then be possible to rapidly advance RNAi therapeutics against potentially any disease target in clinical studies and to thereby treat disease in a new manner. In the near future, the ongoing clinical trials with siRNAs for macular degeneration and RSV may reveal the exciting potential of RNAi therapeutics as the next major class of drug molecules.

### ACKNOWLEDGMENTS

We thank J. Maraganore and B. Greene for critical reading of the manuscript, M. Duckman for graphics assistance and A. Capobianco for word-processing assistance.

### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests (see the *Nature Chemical Biology* website for details).

Published online at <http://www.nature.com/naturechemicalbiology>  
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Dykxhoorn, D.M., Palliser, D. & Lieberman, J. The silent treatment: siRNAs as small molecule drugs. *Gene Ther.* 13, 541–552 (2006).

2. Dykxhoorn, D.M. & Lieberman, J. Running interference: prospects and obstacles to using small interfering RNAs as small molecule drugs. *Annu. Rev. Biomed. Eng.* 8, 377–402 (2006).
3. Rand, T.A., Ginalski, K., Grishin, N.V. & Wang, X. Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proc. Natl. Acad. Sci. USA* 101, 14385–14389 (2004).
4. Ma, J.B. et al. Structural basis for 5'-end-specific recognition of guide RNA by the *A. fuligatus* Piwi protein. *Nature* 434, 666–670 (2005).
5. Matranga, C., Tomari, Y., Shin, C., Bartel, D.P. & Zamore, P.D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 123, 607–620 (2005).
6. Rand, T.A., Petersen, S., Du, F. & Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 123, 621–629 (2005).
7. Soutschek, J. et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178 (2004).
8. Vornlocher, H.P., Zimmerman, T.S., Manoharan, M., Rajeev, K.G., Roehl, I. & Akinc, A. Nuclease-resistant double-stranded RNA for RNA interference. Patent Cooperation Treaty International Application WO2005115481 (2005).
9. Li, B. et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat. Med.* 11, 944–951 (2005).
10. Choung, S., Kim, Y.J., Kim, S., Park, H.O. & Choi, Y.C. Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem. Biophys. Res. Commun.* 342, 919–927 (2006).
11. Allerson, C.R. et al. Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J. Med. Chem.* 48, 901–904 (2005).
12. Prakash, T.P. et al. Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J. Med. Chem.* 48, 4247–4253 (2005).
13. Hoshika, S., Minakawa, N. & Matsuda, A. Synthesis and physical and physiological properties of 4'-thioRNA: application to post-modification of RNA aptamer toward NF- $\kappa$ B. *Nucleic Acids Res.* 32, 3815–3825 (2004).
14. Haeberli, P., Berger, I., Palian, P.S. & Egli, M. Syntheses of 4'-thioribonucleosides and thermodynamic stability and crystal structure of RNA oligomers with incorporated 4'-thiocytosine. *Nucleic Acids Res.* 33, 3965–3975 (2005).
15. Dande, P. et al. Improving RNA interference in mammalian cells by 4'-thio-modified small interfering RNA (siRNA): effect on siRNA activity and nuclease stability when used in combination with 2'-O-alkyl modifications. *J. Med. Chem.* 49, 1624–1634 (2006).
16. Prakash, T.P. et al. RNA interference by 2',5'-linked nucleic acid duplexes in mammalian cells. *Bioorg. Med. Chem. Lett.* 16, 3238–3240 (2006).
17. Hall, A.H., Wan, J., Shaughnessy, E.E., Ransay Shaw, B. & Alexander, K.A. RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic Acids Res.* 32, 5991–6000 (2004).
18. Hall, A.H. et al. High potency silencing by single-stranded boranophosphate siRNA. *Nucleic Acids Res.* 34, 2773–2781 (2006).
19. Xia, J. et al. Gene silencing activity of siRNAs with a ribo-difluorotolyl nucleotide. *ACS Chem. Biol.* 1, 176–183 (2006).
20. Somoza, A., Chelliserrykattil, J. & Kool, E.T. The roles of hydrogen bonding and sterics in RNA interference. *Angew. Chem. Int. Edn. Engl.* 45, 4994–4997 (2006).
21. Elbashir, S.M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498 (2001).
22. Elbashir, S.M., Lendecker, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200 (2001).
23. Boese, Q. et al. Mechanistic insights aid computational short interfering RNA design. *Methods Enzymol.* 392, 73–96 (2005).
24. Yuan, B., Latek, R., Hossbach, M., Tuschl, T. & Lewitter, F. siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucleic Acids Res.* 32, W130–W134 (2004).
25. Schwarz, D.S. et al. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199–208 (2003).
26. Khvorova, A., Reynolds, A. & Jayasena, S.D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115, 209–216 (2003).
27. Reynolds, A. et al. Rational siRNA design for RNA interference. *Nat. Biotechnol.* 22, 326–330 (2004).
28. Jackson, A.L. et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635–637 (2003).
29. Lin, X. et al. siRNA-mediated off-target gene silencing triggered by a 7 nt complementarity. *Nucleic Acids Res.* 33, 4527–4535 (2005).
30. Qiu, S., Adema, C.M. & Lane, T. A computational study of off-target effects of RNA interference. *Nucleic Acids Res.* 33, 1834–1847 (2005).
31. Jackson, A.L. et al. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* 12, 1179–1187 (2006).
32. Birmingham, A. et al. 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* 3, 199–204 (2006).
33. Lewis, B.P., Burge, C.B. & Bartel, D.P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20 (2005).
34. Jackson, A.L. et al. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* 12, 1197–1205 (2006).
35. Schwarz, D.S. et al. Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genetics* (in the press).
36. Hornung, V. et al. Sequence-specific potent induction of IFN- $\alpha$  by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* 11, 263–270 (2005).
37. Boggs, R.T. et al. Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucleic Acid Drug Dev.* 7, 461–471 (1997).
38. Hemmi, H. et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745 (2000).
39. Judge, A.D. et al. Sequence-dependent stimulation of the mammalian innate immune system by synthetic siRNA. *Nat. Biotechnol.* 23, 457–462 (2005).
40. Fedorov, Y. et al. Different delivery methods—different expression profiles. *Nat. Methods* 2, 241 (2005).
41. Reynolds, A. et al. Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 12, 988–993 (2006).
42. Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* 11, 50–55 (2005).
43. Palliser, D. et al. An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 439, 89–94 (2005).
44. Shen, J. et al. Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Ther.* 13, 225–234 (2006).
45. Reich, S.J. et al. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* 9, 210–216 (2003).
46. Nakamura, H. et al. RNA interference targeting transforming growth factor- $\beta$  type II receptor suppresses ocular inflammation and fibrosis. *Mol. Vis.* 10, 703–711 (2004).
47. Makimura, H., Mizuno, T.M., Mastaitis, J.W., Agami, R. & Mobbs, C.V. Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci.* 3, 18 (2002).
48. Thakker, D.R. et al. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl. Acad. Sci. USA* 101, 17270–17275 (2004).
49. Thakker, D.R. et al. siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol. Psychiatry* 10, 782–789, 714 (2005).
50. Dorn, G. et al. siRNA relieves chronic neuropathic pain. *Nucleic Acids Res.* 32, e49 (2004).
51. Tan, P.H., Yang, L.C., Shih, H.C., Lan, K.C. & Cheng, J.T. Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Ther.* 12, 59–66 (2005).
52. Luo, M.C. et al. An efficient intrathecal delivery of small interfering RNA to the spinal cord and peripheral neurons. *Mol. Pain* 1, 29 (2005).
53. Salahpour, A., Medvedev, I.O., Beaulieu, J.-M., Gafni, D.R. & Caron, M.G. Local knockdown of genes in the brain using small interfering RNA: a phenotypic comparison with knockout animals. *Biol. Psychiatry*, published online 17 May 2006.
54. Kumar, P., Lee, S.K., Shankar, P. & Manjunath, N. A single siRNA suppresses fatal encephalitis induced by two different flaviviruses. *PLoS Med.* 3, e96 (2006).
55. Chen, Y. et al. Adenovirus-mediated small-interference RNA for in vivo silencing of angiotensin AT1a receptors in mouse brain. *Hypertension* 47, 230–237 (2006).
56. Xia, H. et al. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar atrophy. *Nat. Med.* 10, 816–820 (2004).
57. Harper, S.Q. et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc. Natl. Acad. Sci. USA* 102, 5820–5825 (2005).
58. Rodriguez-Lebron, E., Denovan-Wright, E.M., Nash, K., Lewin, A.S. & Mandel, R.J. Intrastriatal AAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol. Ther.* 12, 618–633 (2005).
59. Ralph, G.S. et al. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat. Med.* 11, 429–433 (2005).
60. Raoul, C. et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat. Med.* 11, 423–428 (2005).
61. Singer, O. et al. Targeting BACE1 with siRNAs ameliorates Alzheimer disease neurodegeneration in a transgenic model. *Nat. Neurosci.* 8, 1343–1349 (2005).
62. Niu, X.Y., Peng, Z.L., Duan, W.Q., Wang, H. & Wang, P. Inhibition of HPV 16 E6 oncogene expression by RNA interference in vitro and in vivo. *Int. J. Gynecol. Cancer* 16, 743–751 (2006).
63. Grzelinski, M. et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts. *Hum. Gene Ther.* 17, 751–766 (2006).
64. Kim, W.J. et al. Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol. Ther.* 14, 343–350 (2006).
65. Song, E. et al. Antibody-mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* 23, 709–717 (2005).
66. Takei, Y., Kadomatsu, K., Yuzawa, Y., Matsuo, S. & Muramatsu, T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res.* 64, 3365–3370 (2004).
67. Minakuchi, Y. et al. Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. *Nucleic Acids Res.* 32, e109 (2004).
68. McNamara, J.O. et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* 24, 1005–1015 (2006).
69. Gurzov, E.N. & Izquierdo, M. RNA interference against Hec1 inhibits tumor growth in vivo. *Gene Ther.* 13, 1–7 (2005).
70. Pulkuri, S.M. et al. RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo. *J. Biol. Chem.* 280, 36529–36540 (2005).

71. Zhang, Y. *et al.* Engineering mucosal RNA interference in vivo. *Mol. Ther.* 14, 336–342 (2006).

72. Shankar, P., Manjunath, N. & Lieberman, J. The prospect of silencing disease using RNA interference. *J. Am. Med. Assoc.* 293, 1367–1373 (2005).

73. Zimmermann, T.S. *et al.* RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111–114 (2006).

74. Morrissey, D.V. *et al.* Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* 41, 1349–1356 (2005).

75. Geisbert, T.W. *et al.* Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 193, 1650–1657 (2006).

76. Takeshita, F. & Ochiya, T. Therapeutic potential of RNA interference against cancer. *Cancer Sci.* 97, 689–696 (2006).

77. Pal, A. *et al.* Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. *Int. J. Oncol.* 26, 1087–1091 (2005).

78. Schiffelers, R.M. *et al.* Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* 32, e149 (2004).

79. Urban-Klein, B., Werth, S., Abuharbeid, S., Czubayko, F. & Aigner, A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther.* 12, 451–466 (2005).

80. Hu-Lieskovak, S., Heidel, J.D., Bartlett, D.W., Davis, M.E. & Triche, T.J. Sequence-specific knockdown of EWS-FL1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* 65, 8984–8992 (2005).

81. Grimm, D. *et al.* Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441, 537–541 (2006).

82. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811 (1998).

## Comparison of the Suppressive Effects of Antisense Oligonucleotides and siRNAs Directed Against the Same Targets in Mammalian Cells

MAKOTO MIYAGISHI,<sup>1,2</sup> MARIKO HAYASHI,<sup>1</sup> and KAZUNARI TAIRA<sup>1,2</sup>

### ABSTRACT

RNA interference appears to be a potentially powerful tool for studies of genes of unknown function. However, differences in efficacy at different target sites remain problematic when small interfering RNA (siRNA) is used as an effector. Similar problems are associated with attempts at gene inactivation using antisense oligonucleotides (ODNs) and ribozymes. We performed a comparative analysis of the suppressive effects of three knockdown methods, namely, methods based on RNA interference (RNAi), antisense ODNs, and ribozymes, using a luciferase reporter system. Dose-response experiments revealed that the  $IC_{50}$  value for the siRNA was about 100-fold lower than that of the antisense ODN. Our results provide useful information about the positional effects in RNAi, which might help to improve the design of effective siRNAs.

### INTRODUCTION

THE DRAFT SEQUENCE OF THE HUMAN GENOME (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001) includes many genes of unknown function, and identification of these genes is of great importance. Methods for disrupting the expression of specific genes are critical for accurate analysis of the functions of unknown genes, and at present, three major tools, namely, antisense oligonucleotides (ODNs), ribozymes, and RNA interference (RNAi), are available for suppression of the expression of individual specific genes.

Antisense ODNs are short pieces of synthetic, chemically modified DNA that are designed to bind to their target mRNA via Watson-Crick base pairing, thereby specifically inhibiting the expression of the target mRNA (Dove, 2002). As opposed to the apparently simple theory, the mechanism of inhibition by antisense ODNs

seems to involve several complicated steps, including inhibition of translation, splicing, or transport of the target mRNA or degradation of the DNA/RNA hybrid by RNase H.

Ribozymes are RNA molecules with enzymatic activities that bind selectively to and cleave specific target RNAs (Kuwabara et al., 2000a,b; Takagi et al., 2001). It was expected initially that ribozymes might be useful as therapeutic agents and as tools for the functional analysis of genes of interest. However, high-level activities or ribozymes *in vivo* depend on the stability and localization of the transcript, the cleavage activity, and the accessibility of the target RNA to the ribozyme. Nonetheless, a chimeric tRNA<sup>Val</sup> ribozyme, transcribed at a high level from a pol III promoter, with considerable stability in cells and the capacity for translocation to the cytoplasm, has yielded many successful results in cultured cells and animals (Kato et al., 2001; Koseki et al., 1999; Tanabe et al., 2000).

<sup>1</sup>Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Tokyo 113-8656, Japan.

<sup>2</sup>Gene Function Research Laboratory, National Institute of Advanced Industrial Science and Technology (NIAIST), Tsukuba Science City 305-8562, Japan.

RNAi is a recently discovered knockdown phenomenon that is induced by double-stranded RNA (dsRNA) and that has been demonstrated in plants, nematodes, *Drosophila*, protozoa, and mammalian cells (Fire et al., 1998; Fire, 1999; Hammond et al., 2001b; Sharp, 2001; Zamore, 2001; Caplen et al., 2001; Elbashir et al., 2001a). The mechanism of RNAi is not fully understood, but recent genetic and biochemical studies have revealed some details at the molecular level. Inside the cell, dsRNAs are digested into fragments of 21–23 nucleotides (nt), with a 2-nt 3'-overhang, by an RNase III-related enzyme, designated Dicer (Bernstein et al., 2001). Subsequently, these small fragments, known as small interfering RNAs (siRNAs), are incorporated into the RNA-induced silencing complex (RISC) (Hammond et al., 2001a; Nykanen et al., 2001). The active complexes containing the guide RNA recognize and cleave the target RNA.

The activity of siRNA is high even at low concentrations, without apparent toxicity. However, recent reports suggest that the efficacy of siRNA exhibits serious dependence on the target site, as is the case for antisense ODNs and ribozymes, which might significantly limit the conve-

nient use of siRNA (Holen et al., 2002; Miyagishi and Taira, 2002). In the present study, we compared the efficacy of suppression and positional dependency of these knockdown methods, using antisense ODNs and RNAi.

## MATERIALS AND METHODS

### *Cell culture, transfections, and assays of expression of reporter genes*

HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed with Lipofectamine™ 2000 reagent (Life Technologies, Rockville, MD) using 1 nM siRNA or 100 nM ribozymes, as described by the manufacturer. For experiments with antisense ODNs, cells were transfected with 30 ng of an RSV-Renilla luciferase plasmid (pRL-RSV) (Miyagishi et al., 2000), 30 ng of a firefly luciferase expression vector pGL3 (Promega, Madison, WI) using FuGENE 6 (Roche Applied Science, Mannheim, Germany), and 200 nM antisense ODNs using Oligofectamine™

Site 1	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-CUUACGCGUGAGUACUUCGAAU-3' 5'-UCGAAGUACUCAGCGUAAGUU-3' 5'-CATTTCGAAGTACTCAGCGT 5'-GGUUGCAACCUGAUGAGGCCGA AAGGCCGAAACUCAGCGUAAG-3'
Site 2	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UUCGUCACAUCAUCUACUU-3' 5'-GUAGAUGAGAUGAGACGAAUU-3' 5'-GGAGGTAGATGAGATGTGAC 5'-GGGGUAGAACUGAUGAGGCCGA AAGGCCGAAAGAUGUGA-3'
Site 3	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UGCCAGAGUCCUUCGGAUAGGG-3' 5'-CUAUCGAAGGACUCUGGCACA-3' 5'-CCTATCGAAGGACTCTGGCA-3' 5'-GGATCGAAGCTGATGAGGCCGA AGGCCGAAAATCTGGC-3'
Site 4	siRNA(sense) siRNA(antisense) Antisense ODN	5'-GUGCGCUGCGUGGUGCCAACUU-3' 5'-GUUGGCACCAAGCAGCGCACUU-3' 5'-TTGGCACCAAGCAGCGCACUU-3'
Site 5	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UAAGGAAGUCGGGGAAAGCGGU-3' 5'-CGCUUCCCCGACUUCUUAAG-3' 5'-CCGCTTCCCCGACTTCCTTA-3' 5'-GGCUUCCCCCUGAUGAGGCCGA AAGCCGAAACUUCUU-3'
Site 6	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-CAGGUUGGCUCCCCGCUGAAUUG-3' 5'-AUUCAGCGGGAGCCACCUGAU-3' 5'-AATTCAAGCGGGAGCCAGGTG-3' 5'-GGUCAGCGGCUGAUGAGGCCGA AAGGCCGAAAGCCACCU-3'

FIG. 1. Design of siRNAs and antisense ODNs targeted to six sites in the firefly gene for luciferase.

(Life Technologies) in Optimem medium (Life Technologies). Three hours after transfections, the medium was replaced by serum-containing medium. Luciferase activities were analyzed after 24 hours with the dual-luciferase system (Promega).

#### RNA and antisense ODNs

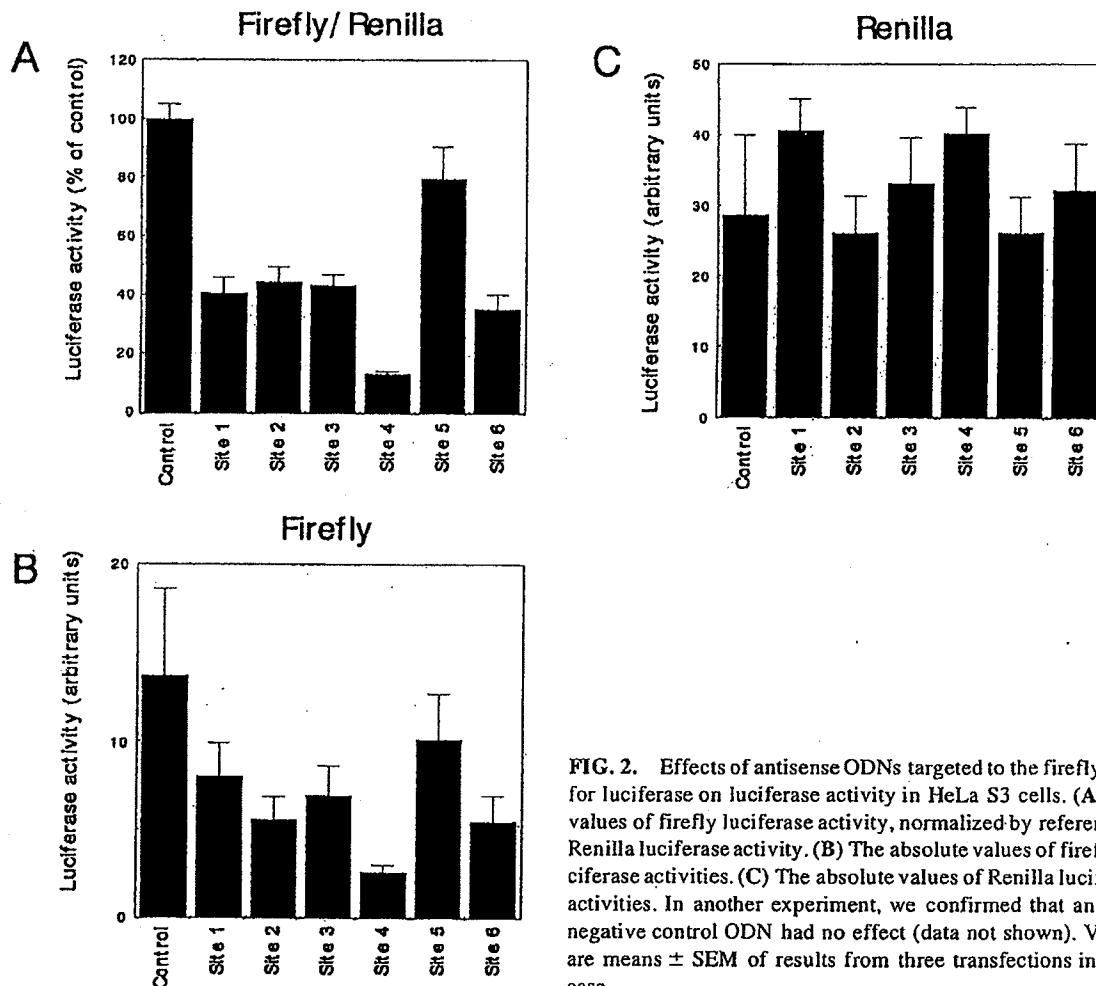
RNA ODNs were synthesized by an automated synthesizer (model 394) (Applied Biosystems, Foster City, CA). The synthetic RNAs were deprotected and purified by electrophoresis on a denaturing polyacrylamide gel. After elution from the gel, the RNAs were desalted by passage through a NAP-10 column (Amersham Pharmacia Biotech, Piscataway, NJ) in RNase-free water. Fractions were reduced to dryness under a vacuum, and residues were resuspended in annealing buffer (phosphate-buffered saline [PBS], pH 6.8, 2 mM MgCl<sub>2</sub>). RNA oligonucleotides (10  $\mu$ M) were annealed by incubation at 95°C for 1 minute, cooled to 70°C, and subse-

quent slowly cooled to 4°C for 2 hours. The sequences of the sense and antisense strands of negative control siRNA were follows: sense, 5'-CUU UCA GCU UCG AUG UAG GTT-3', antisense, 5'-CCU ACA UCG AAG CUG AAA GTT-3'. HPLC-purified phosphorothioate ODNs were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The sequence of the negative control antisense ODN was 5'-CCA ATG TCA AGC ACT TCC GTT-3'. Ribozymes were synthesized from annealed synthetic ODNs by transcription from a T7 promoter using MEGASHortscript™ T7 kit (Ambion, Austin, TX) and purified by electrophoresis on a urea-polyacrylamide gel (7 M urea, 20% polyacrylamide).

## RESULTS

#### Selection of target sites in firefly gene for luciferase

We used a dual-luciferase assay system to evaluate the suppressive effects of the three knockdown methods, us-



**FIG. 2.** Effects of antisense ODNs targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. (A) The values of firefly luciferase activity, normalized by reference to Renilla luciferase activity. (B) The absolute values of firefly luciferase activities. (C) The absolute values of Renilla luciferase activities. In another experiment, we confirmed that another, negative control ODN had no effect (data not shown). Values are means  $\pm$  SEM of results from three transfections in each case.

ing synthetic phosphorothioated antisense ODNs, synthetic siRNAs, and synthetic ribozymes. In this system, we measured firefly and Renilla luciferase activities in the same sample, and suppressive effects on the expression of firefly luciferase were standardized by reference to the expression of Renilla luciferase, which allowed accurate evaluation of the suppressive effects. We selected six target sites within the coding region of the firefly gene for luciferase. Each target sequence included the NUX triplet motif at the center of the sequence, as this is the motif at which cleavage by the hammerhead ribozyme occurs. N can be any residue, and X can be C, U, or A. The length of each arm of the ribozyme was set at 7–9 nt.

In the case of RNAi, the cleavage site for a target RNA has been identified as being 11 nt downstream of the target position that is complementary to the 3'-most nucleotide of the antisense strand of the siRNA (Elbashir et al., 2001a,b). We selected six sites for siRNAs that overlapped the cleavage site as the ribozyme (the cleavage sites were not necessarily identical, but the annealing regions overlapped each other). A length of 21 nt and a 2-nt 3'-overhang were chosen for the siRNA because this form of siRNA was shown to be most effective with *Drosophila* lysate *in vitro* (Elbashir et al., 2001b).

In our antisense experiments, we used 20-nt phosphorothioate-modified oligonucleotides. However, as we used ribozymes as controls for the stability of siRNAs, we did not chemically modify the ribozymes. We used the naked RNAs. Details of the various sense and antisense siRNAs and antisense ODNs are shown in Figure 1.

#### Suppressive effects of antisense ODNs

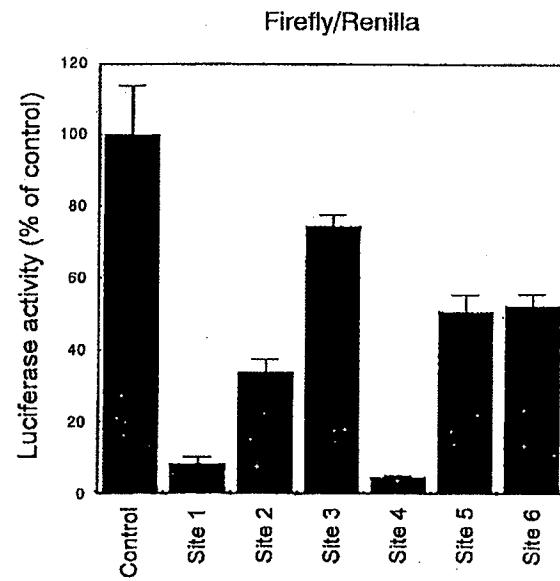
In the first experiment, we examined the effects of antisense ODNs against the gene for firefly luciferase. We cotransfected HeLa S3 cells with a series of antisense ODNs, the firefly luciferase expression vector, and the Renilla luciferase expression vector, using various cationic transfection lipids, including Lipofectin™, Lipofectamine, Lipofectamine 2000, FuGENE 6, and Effectene™. The luciferase assays gave poor results because the activities of Renilla luciferase, used as an internal control, were too low or were unstable when we used antisense ODNs at 200 nM or higher concentrations for transfections. High doses of ODNs might have an inhibitory effect on the efficiency of transfection of plasmids, or they might act nonspecifically to block protein synthesis.

Oligofectamine appeared to be a much better reagent for transfection of antisense ODNs. It is a recently developed low-toxicity reagent that was optimized for delivery of ODNs. We continued to use FuGENE 6 for the transfection of plasmids. We obtained greatly improved luciferase activities, with stable values and low toxicity. Us-

ing this procedure, we evaluated the effects of antisense ODNs targeted to six sites in the firefly gene for luciferase. As shown in Figure 2, cotransfections with antisense ODNs that corresponded to site 1, site 2, site 3, site 5, and site 6 of the firefly gene for luciferase plus the firefly luciferase expression vector and the Renilla luciferase expression vector caused decreases in firefly luciferase activity of 59%, 55%, 57%, 23%, and 65% respectively. The antisense ODN targeted to site 4 had stronger suppressive activity (87%) without any negative effects on Renilla luciferase activity, compared with the negative control (Fig. 2C).

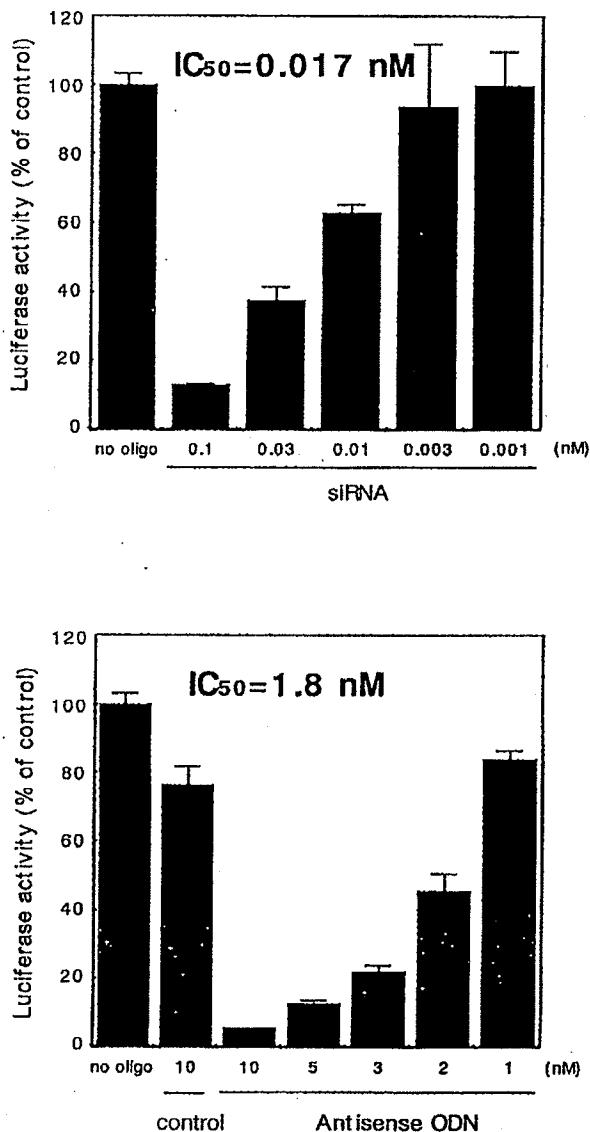
#### Effects of RNAi on expression of firefly gene for luciferase

We transfected cells with siRNAs using the Lipofectamine 2000 reagent. This lipid allows cotransfection of HeLa S3 cells with siRNAs and plasmids at high efficiency (Elbashir et al., 2001a; Miyagishi and Taira, 2002). We examined the suppressive activities of siRNAs that corresponded to the six sites in the firefly gene for luciferase and found that the siRNAs directed against site 1 and site 4 strongly suppressed the luciferase activity (92% and 95%), whereas the corresponding val-

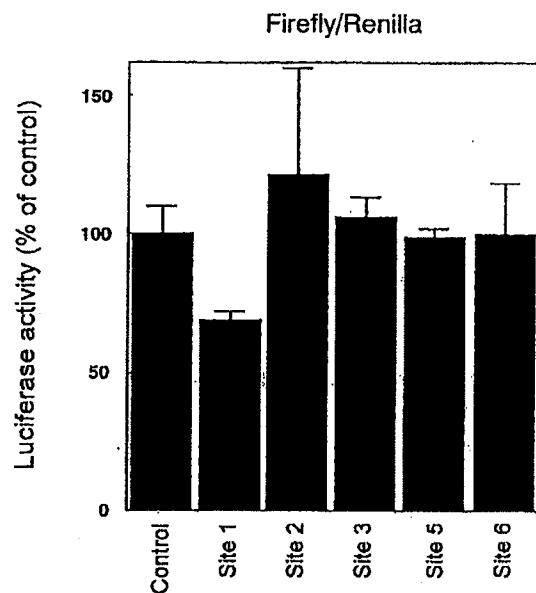


**FIG. 3.** Effects of siRNAs targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and 1 nM siRNA directed against individual target sites in the firefly gene for luciferase or with control siRNA. Values were normalized by reference to Renilla luciferase activity. Values are means  $\pm$  SEM of results from three transfections in each case.

ues for site 2, site 3, site 5, and site 6 were much lower (66%, 25%, 49%, and 48%, respectively) (Fig. 3). The results demonstrated the positional dependence of RNAi, as was also the case for the antisense ODNs. Although the correlation coefficient between the results for antisense ODNs and siRNAs was low (0.42), it is of interest that the highest efficacy was observed at the same posi-



**FIG. 4.** Dose dependence of siRNAs and antisense ODNs on luciferase activity. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and the indicated amounts of siRNA or antisense ODN directed against site 4 in the firefly gene for luciferase or with control siRNA or control antisense ODN. Values are means  $\pm$  SEM of results from three transfections in each case.



**FIG. 5.** Effects of ribozymes targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. Values were normalized by reference to Renilla luciferase activity. Values are means  $\pm$  SEM of results from three transfections in each case.

tion (site 4), even though the two systems are quite different. The low correlation between the antisense ODN and siRNA may originate from the small difference of target sites for siRNA and ODN at single nucleotide level (especially at site 1 and site 2).

To compare the suppression efficacy of two methods, we performed dose-response experiments of the siRNA and the ODN on the suppression effect of luciferase gene expression at site 4. Under this experimental condition, the inhibitory concentration of 50% reduction for luciferase activity ( $IC_{50}$ ) for the siRNA and the ODN is 0.017 nM and 1.8 nM, respectively (Fig. 4). It is important to note that the  $IC_{50}$  value of the siRNA was about 100-fold lower than that of the antisense ODN.

#### Suppressive effects of ribozymes

We examined the effects of unmodified ribozymes directed against the six sites in the firefly gene for luciferase. We demonstrated previously that ribozymes function effectively in cells when transcribed as tRNA constructs under the control of a pol III promoter (Kato et al., 2001; Koseki et al., 1999; Tanabe et al., 2000). However, in the present study, in order to compare the effects of concentration as an index of stability in cells between siRNAs and ribozymes, we introduced naked RNA ribozymes directly into cells, in the same way as we had introduced antisense ODNs and siRNAs, in order to evaluate directly the activities of siRNAs and ribozyme. Ribozymes

were synthesized by T7 polymerase, purified by electrophoresis, and introduced into HeLa S3 cells with the firefly luciferase expression vector and the Renilla luciferase expression vector under the same conditions as those used for siRNA, with the exception of significantly higher final concentrations (100 nM; note that the concentration of siRNA was 1 nM; with Lipofectamine 2000 reagent) (Fig. 5). Even at these higher concentrations, we observe no significant reduction in the expression of firefly luciferase (data not shown).

## DISCUSSION

The dual-luciferase system allows the accurate evaluation of gene-suppressive effects and has been used widely in analyses of the mechanism of RNAi (Elbashir et al., 2001a,b; Miyagishi and Taira, 2002). Nonetheless, there are only a few reports of application of the system to analysis of the effects of antisense ODNs (Faria et al., 2001). In present study, we established a system that allowed us to evaluate antisense effects accurately by use of different cationic lipids for antisense ODNs and reporter plasmids. Our protocol allowed us to compare site-dependent effects between antisense ODNs and siRNAs.

Analysis of the effects of the siRNAs targeted to six sites in the firefly gene for luciferase revealed that positional effects were not well correlated with positional effects on the efficacy of antisense ODNs, but both an siRNA and an antisense ODN were most effective when directed against site 4. This result implies that a major factor in site dependence of the effect of siRNA might be accessibility to the target mRNA, as is also the case for antisense ODNs and ribozymes (Kawasaki et al., 2002; Kawasaki and Taira, 2002; Krupp and Gaur, 2000; Warashina et al., 2001). It is possible that the effects of siRNA and ODN might share some common features. However, we could not find any significant correlation between structures of mRNA, as predicted by Mfold (Zuker and Jacobson, 1998), and the suppressive effects of siRNAs. However, more experimental data about the accessibility of antisense ODN to target RNA *in vitro* and *in vivo* are needed for more precise estimations of such a relationship.

In our experiment with naked ribozymes, we failed to detect suppressive activity even at a concentration of ribozyme of 100 nM during transfection, despite the apparent repressive activity of siRNA under similar conditions at a concentration of 1 nM. This result was most likely due to the poor resistance to nuclease of naked single-stranded ribozymes in cells. By contrast, siRNAs, which are 21–23-nt RNA duplexes with a 2-nt or 3-nt 3'-overhang, are considered to be much more resistant than ribozymes to nucleases. Indeed, we showed previously that an siRNA duplex was significantly more stable in

cells than the cognate single-stranded sense or antisense RNA, with transcription under the control of the identical promoter in each case (Miyagishi and Taira, 2002). Double-stranded siRNAs appear to be protected by RISC from attacks by RNases, whereas ssRNA and ribozymes are not protected by such proteins in cells. Thus, constitutive expression of ribozymes from vectors is required for the effective exploitation of ribozymes.

In our comparative analysis of three major methods for suppression of gene expression, we found a low but significant correlation between the effects of siRNA and antisense ODN. Our results led to the hypothesis that one of the major factors responsible for the dependence of the activity of siRNA on its target site is accessibility to the target mRNA. This working hypothesis should help us focus on such effects and help us to clarify the mechanisms of positional effects in the suppression of gene expression.

## REFERENCES

- BERNSTEIN, E., CAUDY, A.A., HAMMOND, S.M., and HANNON, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.
- CAPLEN, N.J., PARRISH, S., IMANI, F., FIRE, A., and MORGAN, R.A. (2001). Specific inhibition of gene expression by small double-strandedRNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* **98**, 9742–9747.
- DOVE, A. (2002). Antisense and sensibility. *Nat. Biotechnol.* **20**, 121–124.
- ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.
- ELBASHIR, S.M., MARTINEZ, J., PATKANIOWSKA, A., LENDECKEL, W., and TUSCHL, T. (2001b). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888.
- FARIA, M., SPILLER, D.G., DUBERTRET, C., NELSON, J.S., WHITE, M.R., SCHERMAN, D., HÉLÈNE, C., and GIOVANNANGELI, C. (2001). Phosphoramidate oligonucleotides as potent antisense molecules in cells and *in vivo*. *Nat. Biotechnol.* **19**, 40–44.
- FIRE, A. (1999). RNA-triggered gene silencing. *Trends Genet.* **15**, 358–363.
- FIRE, A., XU, S., MONTGOMERY, M.K., KOSTAS, S.A., DRIVER, S.E., and MELLO, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
- HAMMOND, S.M., BOETTCHER, S., CAUDY, A.A., KOBAYASHI, R., and HANNON, G.J. (2001a). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146–1150.
- HAMMOND, S.M., CAUDY, A.A., and HANNON, G.J. (2001b). Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.* **2**, 110–119.

HOLEN, T., AMARZGUIOUI, M., WIIGER, M.T., BABAIE, E., and PRYDZ, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.* **30**, 1757–1766.

INTERNATIONAL HUMAN GENOME SEQUENCING CONSORTIUM. (2001). Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921.

KATO, Y., KUWABARA, T., WARASHINA, M., TODA, H., and TAIRA, K. (2001). Relationships between the activities *in vitro* and *in vivo* of various kinds of ribozyme and their intracellular localization in mammalian cells. *J. Biol. Chem.* **276**, 15378–15385.

KAWASAKI, H., ONUKI, R., SUYAMA, E., and TAIRA, K. (2002). Identification of genes that function in the TNF-alpha-mediated apoptotic pathway using randomized hybrid ribozyme libraries. *Nat. Biotechnol.* **20**, 376–380.

KAWASAKI, H., and TAIRA, K. (2002). Identification of genes by hybrid ribozymes that couple cleavage activity with the unwinding activity of an endogenous RNA helicase. *EMBO Rep.* **3**, 443–450.

KOSEKI, S., TANABE, T., TANI, K., ASANO, S., SHIODA, T., NAGAI, Y., SHIMADA, T., OHKAWA, J., and TAIRA, K. (1999). Factors governing the activity *in vivo* of ribozymes transcribed by RNA polymerase III. *J. Virol.* **73**, 1868–1877.

KRUPP, G., and GAUR, R.K. (2000). *Ribozyme, Biochemistry and Biotechnology*. (Eaton Publishing, Natick, MA).

KUWABARA, T., WARASHINA, M., and TAIRA, K. (2000a). Allosterically controllable ribozymes with biosensor functions. *Curr. Opin. Chem. Biol.* **4**, 669–677.

KUWABARA, T., WARASHINA, M., and TAIRA, K. (2000b). Allosterically controllable maxizymes cleave mRNA with high efficiency and specificity. *Trends Biotechnol.* **18**, 462–468.

MIYAGISHI, M., FUJII, R., HATTA, M., YOSHIDA, E., ARAYA, N., NAGAFUCHI, A., ISHIHARA, S., NAKAJIMA, T., and FUKAMIZU, A. (2000). Regulation of Lef-mediated transcription and p53-dependent pathway by associating  $\beta$ -catenin with CBP/p300. *J. Biol. Chem.* **275**, 35170–35175.

MIYAGISHI, M., FUJII, R., HATTA, M., YOSHIDA, E., ARAYA, N., NAGAFUCHI, A., ISHIHARA, S., NAKAJIMA, T., and FUKAMIZU, A. (2001). Regulation of Lef-mediated transcription and p53-dependent pathway by associating  $\beta$ -catenin with CBP/p300. *J. Biol. Chem.* **275**, 35170–35175.

MIYAGISHI, M., and TAIRA, K. (2002). U6 promoter driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* **20**, 497–500.

NYKANEN, A., HALEY, B., and ZAMORE, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321.

SHARP, P.A. (2001). RNA interference 2001. *Genes Dev.* **15**, 485–490.

TAKAGI, Y., WARASHINA, M., STEC, W.J., YOSHINARA, K., and TAIRA, K. (2001). Survey and summary: Recent advances in the elucidation of the mechanisms of action of ribozymes. *Nucleic Acids Res.* **29**, 1815–1834.

TANABE, T., KUWABARA, T., WARASHINA, M., TANI, K., TAIRA, K., and ASANO, S. (2000). Oncogene inactivation in a mouse model. *Nature* **406**, 473–474.

VENTER, J.C., et al. (2001). The sequence of the human genome. *Science* **291**, 1304–1351.

WARASHINA, M., KUWABARA, T., KATO, Y., SANO, M., and TAIRA, K. (2001). RNA-protein hybrid ribozymes that efficiently cleave any mRNA independently of the structure of the target RNA. *Proc. Natl. Acad. Sci. USA* **98**, 5572–5577.

ZAMORE, P.D. (2001). RNA interference: Listening to the sound of silence. *Nat. Struct. Biol.* **8**, 746–750.

ZUKER, M., and JACOBSON, A.B. (1998). Using reliability information to annotate RNA secondary structures. *RNA* **4**, 669–679.

Address reprint requests to:  
 Professor Kazunari Taira  
 Department of Chemistry and Biotechnology  
 School of Engineering  
 The University of Tokyo  
 Hongo, Tokyo 113-8656  
 Japan

E-mail: taira@chembio.t.u-tokyo.ac.jp

Received June 10, 2002; accepted in revised form  
 November 14, 2002.